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A histological and ultrastructural investigation of the female reproductive system of the water snake (*Erythrolamprus miliaris*): Oviductal cycle and sperm storage

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Abstract

We studied the structural and cellular organisation of the oviduct of *Erythrolamprus miliaris* including its morphological variation during the reproductive cycle using light microscopy, scanning electron microscopy and transmission electron microscopy. Four anatomically distinct regions compose the oviduct of *E. miliaris* including the anterior and posterior infundibulum, glandular uterus, non-glandular uterus and pouch. The cells of the oviductal epithelium secrete material by apocrine and merocrine processes, which vary between the anatomical regions and according to each phase of the reproductive cycle. The infundibular epithelium secretes electron dense vacuoles, which suggests the production of lipids, whereas the epithelial secretion of the glandular uterus, non-glandular uterus and pouch creates lucent and slightly electron dense vacuoles, indicating the production of glycoproteins. The timing of mating, vitellogenesis and sperm storage directly influences the morphofunctional alterations in the oviducts of *E. miliaris*. Sperm storage occurs only in the infundibular receptacles with increased production of the neutral carbohydrates in the presence of male gametes. Sperm storage happens in vitellogenic, non-vitellogenic and pregnant females of *E. miliaris*. Thus, females may be able to produce multiple clutches at different seasons of the year regardless of mating during autumn.

KEYWORDS

Erythrolamprus miliaris, histology, oviduct, sperm storage receptacle, ultrastructure

1 | INTRODUCTION

The reptilian oviduct shows some anatomical variation among different lineages (Blackburn, 1998; Girling, 2002). At the macroscopical level, the oviducts are more elongated in snakes than lizards, and at the microscopical level, the oviducts may show the presence or absence of glands and different types of epithelium in each portion (Blackburn, 1998; Girling, 2002). Despite the existence of different oviductal patterns, the tissue structure is very similar among the clades. From the innermost layer to the outer layer, the oviduct is composed by the epithelium, shell gland, connective tissue, circular muscle, longitudinal muscle and serosa (Barros, Rojas, & Almeida-Santos, 2014b; Girling, 2002; Rojas, Barros, & Almeida-Santos, 2015). However, it is important to highlight that there are different proposals of nomenclature for the oviductal regions in snakes (Blackburn, 1998; Rojas et al., 2015; Siegel, Miralles, Chabarria, & Aldridge, 2011; Siegel & Sever, 2008a,b). For example, the infundibulum may be divided into anterior and posterior and the uterus in glandular and non-glandular portions (Blackburn, 1998; Siegel & Sever, 2008a,b). Siegel et al. (2011) also suggest the use of the term "pouch" to characterise the most caudal oviductal portion, previously known as the Giacomini's diverticulum

or vagina. Recently, Rojas et al. (2015) proposed an adjustment in the nomenclature of the oviduct, suggesting the substitution of non-glandular uterus for utero-vaginal junction (UVJ). This term has already been used for other squamate reptiles (Sever & Hamlett, 2002).

One of the most studied functions of the reptilian oviduct is the capacity to store sperm in specific sites of the female reproductive tract, which confers a diversity of reproductive advantages for some species (Almeida-Santos & Salomão, 1997; Schuett, 1992). Sperm storage allows the maintenance of sperm viability until the timing of ovulation in species that do not show synchronous mating and ovulation (Schuett, 1992). There are three different sperm storage sites in the female reproductive tract of snakes: the posterior infundibulum, non-glandular uterus or UVJ and vagina or pouch (Barros, Rojas, & Almeida-Santos, 2014a; Barros et al., 2014b;Barros, Sueiro, & Almeida-Santos, 2012; Fox, 1956, 1963; Halpert, Garstka, & Crews, 1982; Loebens, Rojas, Almeida-Santos, & Cechin, 2017; Rojas et al., 2015; Saint-Girons, 1962; Sever & Hamlett, 2002; Siegel et al., 2011).

During the phases of the reproductive cycle, the oviduct shows ultrastructural alterations of cell's shape and size and the type of secretion they produce (Rojas et al., 2015; Sever & Ryan, 1999; Sever, Ryan, Morris, Patton, & Swafford, 2000; Siegel & Sever, 2008a,b). Cellular changes in oviductal cells are associated to variations in follicular development (vitellogenesis), mating, pregnancy and sperm storage (Heulin et al., 2005; Hoffman & Wimsatt, 1972; Perkins & Palmer, 1996; Siegel & Sever, 2008a,b). An increase in the activity of Golgi complex, endoplasmic reticulum (rough and smooth) with the presence of apical vacuoles (highly electrodenses) and lipid droplets in the cytoplasm occurs during sperm storage (Girling, Cree, & Guillette, 1997; Sever & Hopkins, 2004; Siegel & Sever, 2008a,b). Sperm may also remain embedded inter- or intracellularly in the receptacles during the storage (Sever & Ryan, 1999; Sever et al., 2000; Siegel & Sever, 2008a,b).

There are few studies on the ultrastructure of snake oviducts (Sever & Ryan, 1999; Sever et al., 2000; Siegel & Sever, 2008a,b) and almost none for the Neotropical region. The aim of this study was to provide a histological and cytological characterisation of the oviduct of *Erythrolamprus miliaris* (Linnaeus, 1758) considering the different phases of the reproductive cycle and establishing a comparison within other snake species. It includes an investigation on occurrence of sperm storage in the female reproductive tract for this species. *Erythrolamprus miliaris* is an oviparous snake belonging to Dipsadidae (Xenodontinae) (Grazziotin et al., 2012; Zaher et al., 2009) and widely distributed in South America (Dixon, 1983). Here, we studied the population from south-eastern Brazil, which has a seasonal reproductive cycle with vitellogenesis and oviposition occurring mainly from September to February (Pizzatto & Marques, 2006).

2 | MATERIALS AND METHODS

2.1 | Specimens

We examined a total of 21 mature specimens of *E. miliaris* from the state of São Paulo (south-eastern region of Brazil—23°09′ to 23°27′S; 46°31′ to 47°03′W). Females were considered mature when the diameter of ovarian follicle was >10 mm or if they had oviductal eggs (Pizzatto & Marques, 2006). The number of specimens sampled per months was as follows: January ($n = 5$), February ($n = 4$), April ($n = 2$), May ($n = 2$), July ($n = 1$), August ($n = 2$), September ($n = 1$), October ($n = 1$), November ($n = 2$) and December ($n = 1$). Individuals were euthanised by an intracoelomic injection of thionembutal (30 mg/kg), followed by a 0.2 ml intracardiac injection of potassium chloride (KCl) (Rojas, Barros, & Almeida-Santos, 2013; Rojas et al., 2015). The left side of the reproductive tract was removed and fixed in 4% paraformaldehyde solution, for no less than 48 hr, for light microscopy. Portions of the right oviduct were removed and fixed in Karnovsky's solution and 2.5% glutaraldehyde, for examination with transmission electron microscopy (TEM) and scanning electron microscopy (SEM), respectively. This work is in agreement with the Ethical Principles in Animal Research, adopted by the Brazilian College of Animal experimentation, and it was approved by the Ethical Committee for Animal Research of Butantan Institute (protocol number 668/09).

2.2 | Light microscopy methods

Samples were processed by historesin (Glycol methacrylate—Leica) and paraffin (Petrobrás) methods. Sections 2 and 5 μm thick were cut for historesin and paraffin, respectively. Histological staining methods used were haematoxylin and eosin (H&E) in paraffin (Junqueira, Bignolas, & Brentani, 1979) and toluidine blue–fuchsin (T&F) for sections in historesin (Junqueira, 1995). To determine possible variations in the secretory activity of the oviducts during different phases of the reproductive cycle, including the sperm storage process, the sections were submitted to the following histochemical reactions: periodic acid–Schiff (PAS) for identification of neutral carbohydrates and alcian blue (AB) pH 2.5 for carboxylated glycosaminoglycans. A coomassie blue R-250 (CB) procedure also was used to identify production of proteins by shell glands (H. Braz, unpublished data). Slides were viewed using an Olympus BX51 microscope, and images were obtained via Image—Pro Express Olympus Program.

2.3 | Transmission electron microscopy methods

After fixation in Karnovsky solution, sections of the oviduct (0.5 mm) were rinsed (three times for 15 min) in 0.1 M sodium cacodylate buffer (pH 7.2). Tissues were then postfixed in 1% osmium tetroxide for 1 hr and washed again in sodium cacodylate buffer (pH 7.4) three times for 15 min. Next, the tissues immersed in 0.5% uranyl acetate with 13.3% saccharose for 1 hr and dehydrated in increasing concentrations of ethanol (70% X2, 95% X2 and 100% X3). Sections were subsequently cleared twice with propylene oxide (15 min, each), preincluded 1:1 in propylene oxide/epoxy resin for 3 hr and after in pure epoxy resin "overnight". The final step consisted of inserting tissues in small plain moulds with pure epoxy resin for 72 hr, at 60°C. Once hardened, the blocks were cut at a thickness of 960 nm via a glass knife on a Sorvall MT6000 ultramicrotome and semithin sections were stained with toluidine blue and observed under light microscopy for general orientation. Ultrathin sections (60 nm) through selected regions were obtained with the aid of a diamond knife (DIATOME, Biel, Switzerland) using the same ultramicrotome. Sections were placed on copper grids and stained with uranyl acetate and lead citrate. Grids were then viewed with a LEO 906E Zeiss transmission electron microscope (Leo Electron Microscopy Ltd Corporation Zeiss Leica, Cambridge, England), operating at 80 kV.

2.4 | Scanning electron microscopy methods

The tissues were fixed in 2.5% glutaraldehyde, rinsed in 0.1 M sodium phosphate buffer (pH 7.2) and then postfixed for 90 min in 1% osmium tetroxide. After a rinse in 0.1 M sodium phosphate buffer (pH 7.2) and distilled water, the tissues were incubated in 1% tannin for 1 hr and dehydrated in a graded series of ethanol (50%, 70%, 90% and 100% for 10 min each). Lastly, the dehydrated tissues were coated with gold and examined with a Zeiss LEO 435 VP scanning electron microscope (Leo Electron Microscopy Ltd Corporation Zeiss Leica, Cambridge, England), operating between 15 and 25 kV.

3 | RESULTS

3.1 | Reproductive conditions

Females of *E. miliaris* showing vitellogenic follicles or eggs in the oviducts occurred in September, October, November, January and February (spring–summer) (Table 1). However, follicle growth and pregnancy during May (autumn) were also observed (Table 1). The beginning of the mating season in autumn was inferred by the presence of sperm in the pouch of two females during April (Table 1). We observed sperm in the four oviductal regions in these females (Table 1). Sperm inside the sperm storage receptacles (SSr) in the infundibulum was observed in January, February, April, May, August, October and December (Table 1). A pregnant female showed stored sperm in the receptacles in autumn (May).

3.2 | Light microscopy

The female reproductive tract of *E. miliaris* is composed of four regions with different histological patterns. From the cranial to the caudal orientation, the oviductal regions include the infundibulum (anterior and posterior), glandular uterus, non-glandular uterus and pouch (Figure 1). Despite the variations within these regions, the oviductal epithelium is composed of ciliated and secretory cells in the entire extension of the oviduct, except for the pouch (Figure 1b inset). The infundibulum is characterised as an extremely folded structure with an anterior and non-glandular portion and a posterior portion composed of tubular ciliated glands interspersed with sperm storage receptacles (SSr) (Figure 1b,c). The absence of glands in the anterior infundibulum is the only difference between it and its posterior portion. The infundibular epithelium varies from cuboidal to columnar according to the season of the year. The receptacles of the posterior infundibulum exhibit squamous to cubic simple epithelium without cilia. These SSr house groups of spermatozoa in parallel alignment with their heads orientated towards the epithelium (Figure 1c). The epithelium of the infundibulum showed intense positive reaction to PAS in autumn and spring (Figure 1d) and to AB only in spring (Figure 1e). During sperm storage, we observed intense positivity to PAS in the cytoplasm of tubular ciliated glands and SSr, while a positive reaction to AB was observed only for the apical border of receptacles (Figure 1f,g). The glandular uterus is constituted by a thicker lamina propria than the infundibulum with the presence of simple tubular uterine glands (shell glands) (Figure 1h). This oviductal region has a simple cuboidal epithelium composed mainly of secretory cells but also by some ciliated cells (Figure 1h). The luminal epithelium exhibits intense positivity to PAS during autumn and spring (Figure 2a). However, the PAS reaction was less intense than that for the basement membrane of the uterine epithelium (Figure 2a). Shell glands showed positive reaction to PAS and CB solely in spring, and negative reaction to AB throughout the reproductive cycle (Figure 2a,b). The non-glandular uterus is characterised by long folds and deep furrows or crypts (Figure 2c) and an epithelium pseudostratified with intense reaction to PAS in autumn and spring (Figure 2c inset). The pouch does not have glands in the lamina propria either. The muscular layer of the pouch is better developed than in other oviductal regions

TABLE 1 Specimens used monthly, macroscopic morphometric and sperm abundance

Month	SVL (mm)	Maximum follicle diameter (mm)	Sperm pouch	Sperm NGU	Sperm GU	Sperm SSr
January	755	4.8	N	N	N	
January	692	6.2	N	N	N	N
January[a]	665	5	N	N	N	N
January	535	12	N	N	N	N
January	520	18	N	N	N	N
February	910	10	N	N	N	N
February	878	8	N	N	N	N
February	800	20	N	N	N	
February	735	10	N	N	N	N
April	730	4				
April	605	4				
May	755	15	N	N	N	N
May[a]	460	3	N	N	N	
July	1087	6	N	N	N	N
August	675	5	N	N	N	N
August	725	18	N	N	N	
September	699	17	N	N	N	
October	795	32	N	N	N	
November[a]	695	7	N	N	N	N
November[a]	815	7	N	N	N	N
December	810	7	N	N	N	

GU, glandular uterus; NGU, non-glandular uterus; N, no sperm in the oviduct;  , sperm present in the oviduct.

[a]Gravid female. [Colour figure can be viewed at wileyonlinelibrary.com]

(Figure 2d). The pseudostratified epithelium of the pouch shows positive activity to PAS and AB throughout the year.

3.3 | Electron microscopy

3.3.1 | Infundíbulum

The infundibular epithelium of *E. miliaris* is simple and composed of ciliated and secretory cells (Figure 3a). The ciliated cells have elongated euchromatic nuclei with agglomerates of mitochondria and basal body in the apical region (Figure 3b). Each cilium comes from a basal body with a pattern of microtubules 9 + 2 at the transversal section (Figure 3b inset). During autumn and winter, the secretory cells of the infundibulum exhibit a columnar shape with basal euchromatic nuclei and electron dense vacuoles throughout all the cytoplasm (Figure 3b). The occurrence of rough and smooth endoplasmic reticulum in the secretory cells indicates synthesis activity during these seasons (Figure 3c). During spring and summer, the epithelium shows a decrease in the secretory activity. At that time, a cuboidal shape with heterochromatic nuclei and electron dense vacuoles that can be observed only at the apical portion of the cell characterises the epithelium (Figure 3d). The release of cellular material to the infundibular lumen occurs through an apocrine process during the entire cycle (Figure 3e). Tubular ciliated glands connect the lumen of the posterior infundibulum to the SSr. These tubular glands are composed of two types of cells: some secretory cells and abundant cells with prominent cilia filling the whole lumen (Figure 3f). Both types of cells have heterochromatic nuclei and electron dense vacuoles at the apical portion (Figure 3f). During autumn, the tubular ciliated glands show spermatozoa in the lumen (Figure 4a). The secretory cells of these glands exhibit small electron lucent vacuoles during autumn (Figure 4a inset). A high density of stored sperm in the infundibular receptacles occurs in autumn (Figure 4b).

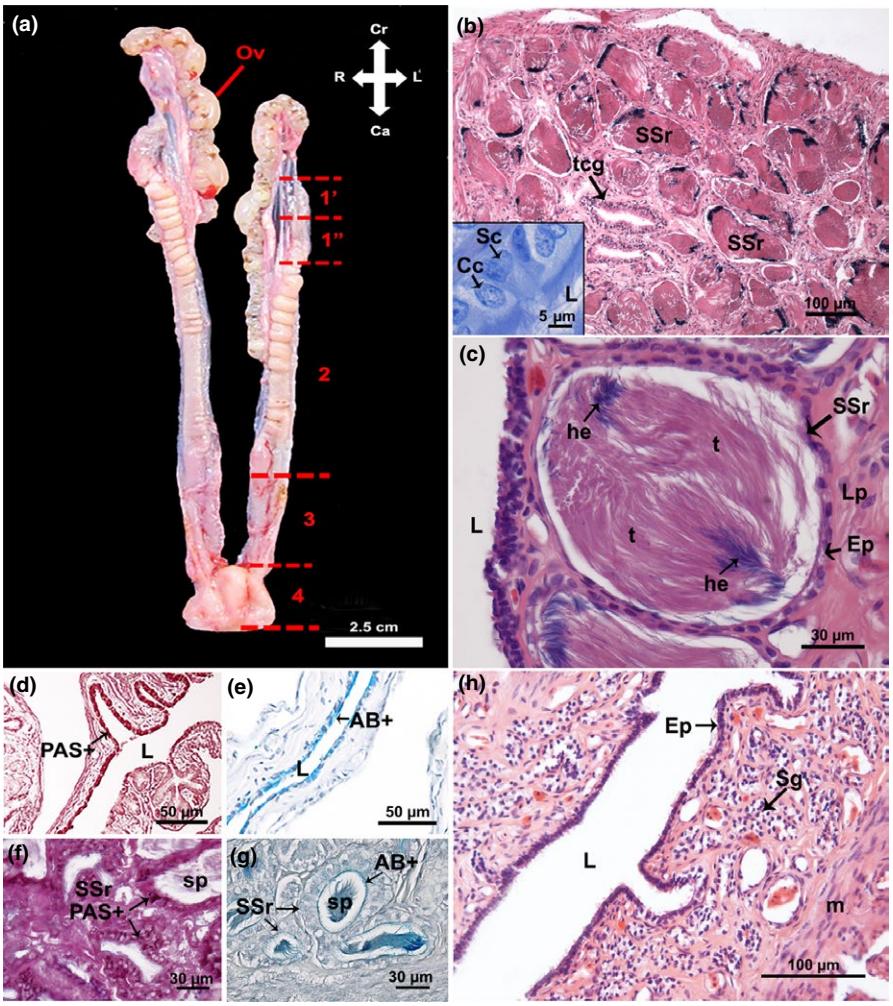

FIGURE 1 Gross morphology and histology of the female reproductive tract of *Erythrolamprus miliaris*. (a) Macroscopic anatomy of the oviduct. (b) Cross section of posterior infundibulum showing tubular ciliated glands and SSr (stained H&E). Inset: the epithelium lining the infundibular lumen shows ciliated and secretory cells (stained T&F). (c) Higher magnification of the SSr (stained H&E). (d) Transverse section of the infundibulum with positive histochemical reactions for PAS during autumn and spring. (e) Infundibular epithelium with positive reaction for AB (pH 2.5) in the spring. (f) SSr with positive reaction for PAS in the presence of the sperm. (g) Apical border of the SSr with positive reaction for AB. (h) Cross section of glandular uterus (stained H&E). 1′, anterior infundibulum; 1″, posterior infundibulum; 2, glandular uterus; 3, non-glandular uterus; 4, pouch; AB+, positive reaction to Alcian Blue; Ca, caudal; Cc, ciliated cells; Cr, cranial; Ep, luminal epithelium; he, spermatozoa head; L, lumen; L′, left; Lp, lamina propria; m, muscularis; Ov, ovaries; PAS+, positive reaction to periodic acid–Schiff; Sc, secretory cells; R, right; sp, spermatozoa; SSr, sperm storage receptacles; t spermatozoa tail; tcg, tubular ciliated glands; Sg, shell glands. [Colour figure can be viewed at wileyonlinelibrary.com]

3.3.2 | Glandular uterus

The epithelium of the glandular uterus is characterised mainly of secretory cells but also by some ciliated cells (Figure 4c,d). The secretory cells exhibit heterochromatic nuclei with agglomerates of mitochondria in the apical portion of the cell (Figure 4d). During autumn (mating) and spring (vitellogenesis), these cells increase the production of electron lucent vacuoles (Figure 4d). A merocrine process releases the vacuoles produced by these cells (Figure 4d inset). In pregnant females, the secretory cells of the uterine epithelium exhibit heterochromatic nuclei of irregular shape and a smaller density of electron lucent vacuoles than vitellogenic females do (Figure 4e). Shell glands occur in this oviductal portion, and they are separated from each other by collagen fibres and the presence of mastocytes (Figure 4f). Shell glands show cells with a prominent nucleolus, well-developed rough endoplasmic reticulum and the capacity to produce cytoplasmic vacuoles which density varies according to the reproductive condition of the female (Figures 4f and 5a). The production of slight electron dense cytoplasmic vacuoles decreases in autumn and winter in non-vitellogenic females, while it increases in spring in vitellogenic females (Figure 4f). Shell glands exhibit remarkable atrophy in gravid females (Figure 5b).

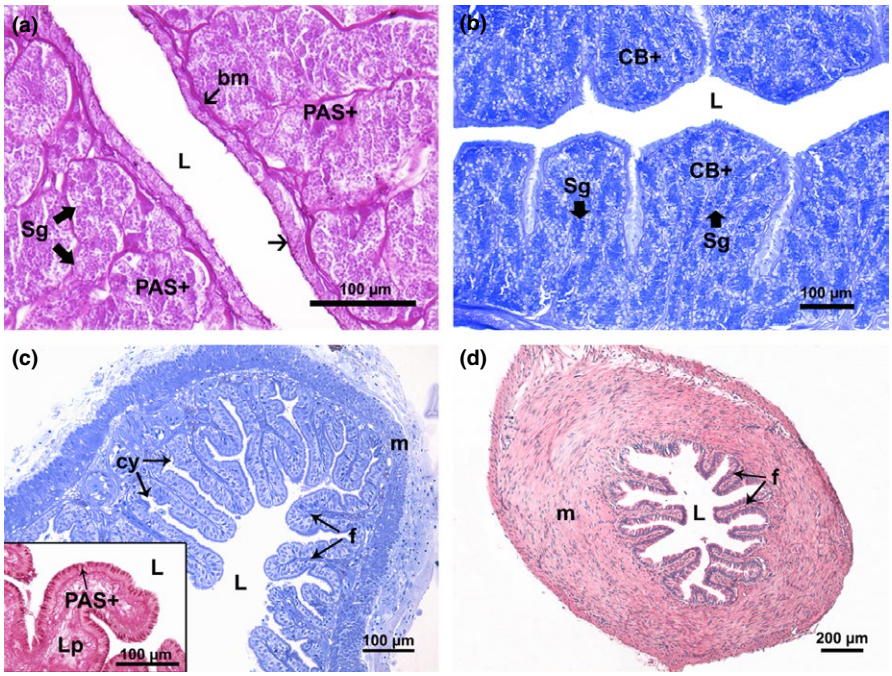

FIGURE 2 Photomicrograph of the glandular uterus, non-glandular uterus and pouch in *Erythrolamprus miliaris*. (a) Transverse section of the glandular uterus with positive histochemical reactions for PAS. (b) Shell glands with positive reactions for CB. (c) Cross section of the non-glandular uterus (stained T&F). Inset: positivity to PAS in the non-glandular uterus in autumn and spring. (d) Transversal section of the pouch (stained H&E). CB+, positive reaction to Coomassie blue; cy, crypts; f, folds; L, lumen; Lp, lamina propria; PAS+, positive reaction to periodic acid–Schiff; Sg, shell glands; (→), apical cytoplasm with positive reaction to PAS. [Colour figure can be viewed at wileyonlinelibrary.com]

3.3.3 | Non-glandular uterus

The epithelium presents a higher concentration of cells with elongated cilia than other oviductal regions (Figure 5c). Ciliated cells are characterised by the high density of mitochondria in the cytoplasm, mainly in the apical region of the cells (Figure 5d). These cells intercalate with few secretory cells separated by intercellular canaliculi (Figure 5d,e). During autumn and spring, we observed secretory cells characterised by elongated euchromatic basal nuclei, cytoplasm with vacuoles filled with flocculent material (electron lucent) and many mitochondria in the apical region (Figure 5d,e). Secretory cells exhibit well-developed Golgi complex in these seasons (Figure 5e), and the flocculent material is released by a merocrine process (Figure 5d). During winter and summer (females with shelled eggs), the synthesis of electron lucent vacuoles decreases in secretory cells. During April (autumn), we observed the presence of sperm in the lumen of the non-glandular uterus (Figure 5f).

3.3.4 | Pouch

This region is constituted by a epithelium with columnar secretory cells and high density of desmosomes between the cells (Figure 6a inset). Secretory cells exhibit euchromatic basal nuclei with vacuoles that almost fill the cytoplasm (Figure 6a). Most vacuoles produced by secretory cells are electron lucent, but some are slightly electron dense during the entire reproductive cycle (Figure 6b). The secretion is released by an apocrine process, and the luminal border of the epithelium is constituted by microvilli (Figure 6b). The epithelial cells of the pouch exhibit a stable pattern of secretion, showing well-developed Golgi complex throughout the year (Figure 6c). Spermatozoa in the pouch lumen were observed in two females collected in April, during autumn (Figure 6d).

4 | DISCUSSION

The oviduct of *E. miliaris* is histologically similar to most snakes studied to date (Barros et al., 2014b; Siegel & Sever, 2008a; Siegel et al., 2011). Here, in this study, we followed Siegel et al. (2011) for oviductal nomenclature to maintain uniformity in the literature. However, the anatomical division of the oviduct is similar to that described for a related species *Philodryas patagoniensis* (Dipsadidae) (Loebens et al., 2017; Rojas et al., 2015). In *E. miliaris,* the nomenclature non-glandular uterus is appropriate because females do not have sperm storage glands or structures in this oviductal portion. We highlight that the term UVJ should be considered, specially for Squamata species that exhibit sperm storage glands in this oviductal portion, as described for *Anolis sagrei* (Dactyloidae) (Sever & Hamlett, 2002; Siegel et al. 2015) and *P. patagoniensis* (Rojas et al., 2015). For the most caudal region of the oviducts, we use the term pouch instead of vagina following Siegel et al. (2011). However, future studies on copulatory adjustment in *E. miliaris* may reveal more specific information about this region and its use as a vagina (see Rojas et al., 2015).

The histological and ultrastructural analysis of the oviduct showed that *E. miliaris* infundibular epithelium is simple just like other dipsadid snakes as *Coniphanes fissidensis* (Siegel et al., 2011) and *P. patagoniensis* (Rojas et al.,

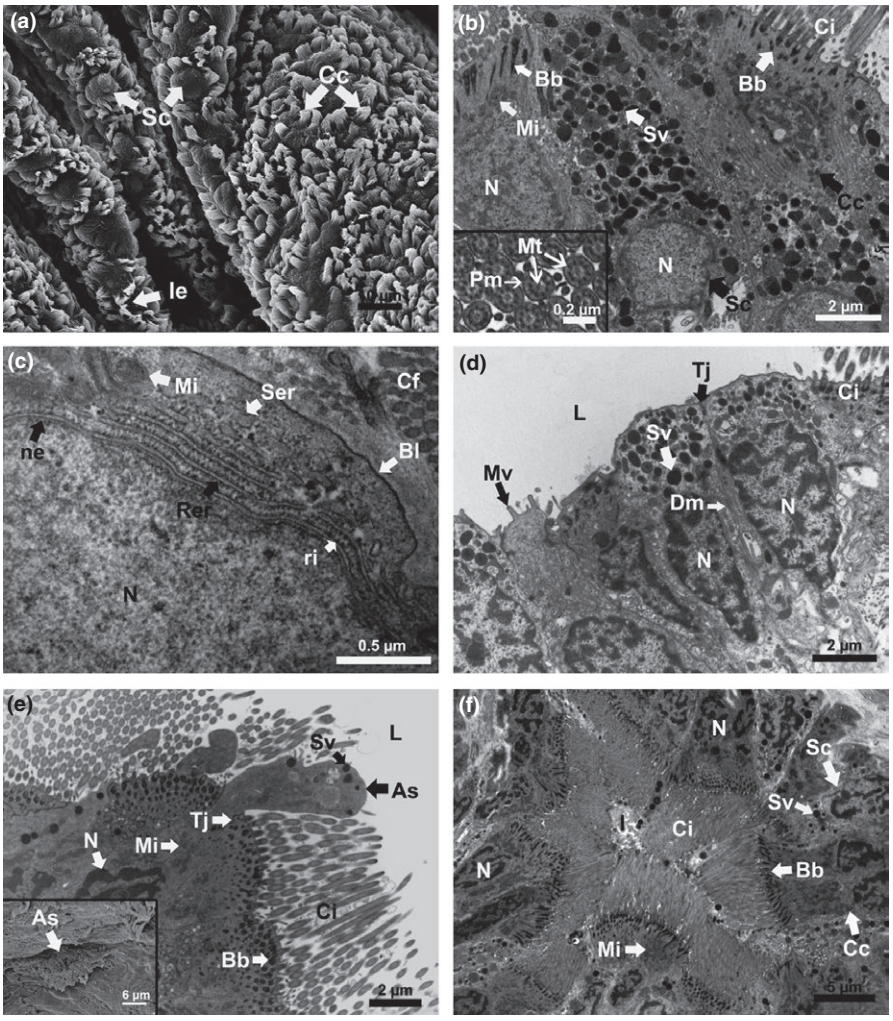

FIGURE 3 Ultrastructure of the posterior infundibulum in female of *Erythrolamprus miliaris* during the reproductive cycle. (a) Scanning electron microscopic of the infundibular epithelium. (b) Transmission electron micrograph of the infundibular epithelium in female throughout autumn–winter. Inset: ciliary ultrastructure. (c) Higher magnification of the organelles present in secretory cells. (d) Infundibular epithelium during spring–summer. (e) Apocrine secretion by infundibular epithelium cells. Inset: scanning electron microscopic of the infundibular epithelium. (f) Overview of the tubular ciliated glands. As, apocrine secretion; Bb, basal bodies; Bl, basal lamina; Cc, ciliated cell; Cf, collagen fibre; Ci, cilia; Dm, desmosome; Ie, infundibular epithelium; L, lumen; Mi, mitochondria; Mt, microtubules; Mv, microvilli; N, nucleus; ne, nuclear envelope; Pm, plasma membrane; Rer, rough endoplasmic reticulum; ri, ribosomes; Sc, secretory cell; Ser, smooth endoplasmic reticulum; Sv, secretory vacuoles; Tj, tight junction

2015), and the natricid *Nerodia sipedon* (Blackburn, 1998). The ciliated cells of the infundibular epithelium are identical in structure to those occurring in the entire oviductal epithelium. The function of ciliated cells is to maintain the movement of mucus and cell debris in the oviduct (Rojas et al., 2015). In addition, they may have a role in the sperm circulation and attraction of spermatozoa to the storage sites (Fox, 1956; Halpert et al., 1982; Hoffman & Wimsatt, 1972). Mitochondria are the most abundant organelle in these ciliated cells, and they are presumably responsible for producing energy to cilia movement. On the other hand, the secretory cells of the infundibulum exhibit well-developed smooth endoplasmic reticulum with the production of electron dense vacuoles, which suggests the secretion of lipids. The increase in these vacuoles occurred mainly during the mating season (autumn), similar to *Seminatrix pygaea* (Natricidae) as observed by Sever et al. (2000). The most abundant organelles in infundibular secretory cells of *Agkistrodon piscivorus* (Viperidae) and *S. pygaea* are mitochondria and smooth endoplasmic reticulum, in addition to the rough endoplasmic reticulum in *S. pygaea* (Sever et al., 2000; Siegel & Sever, 2008*b*; Siegel et al., 2011).

The oviductal epithelium of *E. miliaris* secretes neutral carbohydrates (PAS +) and carboxylated glycosaminoglycans (AB+) in every region of the oviduct, as already described for other squamates (Bauman & Metter, 1977; Fox, 1956; Perkins & Palmer, 1996; Rojas et al., 2015; Saint-Girons, 1975; Sever et al., 2000). The stronger positive reactions to PAS during spring seem to be linked to ovulation in snakes (Palmer & Gillette, 1991; Perkins & Palmer, 1996; Sever & Ryan, 1999; Sever et al., 2000; Siegel & Sever, 2008a,b). These positive reactions in the infundibular epithelium and SSr during autumn suggest the existence of a chemical attraction of spermatozoa to the infundibular region and specifically to the receptacles. The neotropical dipsadid *P. patagoniensis* exhibit this pattern of histochemical reaction (Rojas et al., 2015). The positive reaction to AB indicates the presence of carboxylated glycosaminoglycans whose main function is the retention of water and the maintenance of moisture in the uterine mucosa (Heulin et al., 2005; Perkins & Palmer, 1996; Rojas et al., 2015). Cytoplasmic organelles as the rough endoplasmic reticulum and the production of electron lucent vacuoles by infundibular cells suggest the secretion of glycoproteins.

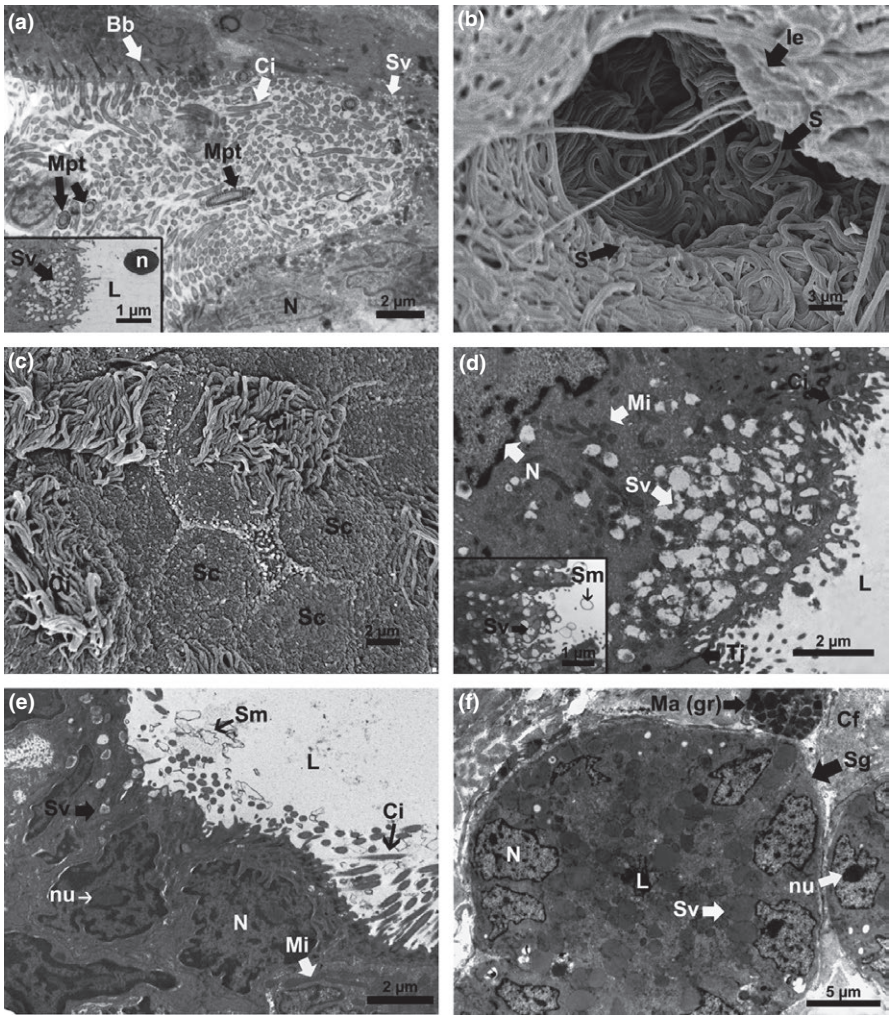

FIGURE 4 Ultrastructure of the posterior infundibulum and seasonal variation of glandular uterus in female of *Erythrolamprus miliaris*. (a) Sperm in the lumen of tubular ciliated glands. (b) Scanning electron microscopic of the SSr in the posterior infundibulum during autumn. (c) Scanning electron microscopic of the glandular uterine epithelium. (d) Transmission electron micrograph of the glandular uterine epithelium during autumn and spring. Inset: merocrine secretion by glandular uterine epithelial cell. (e) Glandular uterine epithelium in pregnant female during summer. (f) Overview of the shell glands with increase in secretory vacuoles in spring. Bb, basal bodies; Cf, collagen fibre; Ci, cilia; Ie, infundibular epithelium; L, lumen; Ma (gr), mastocytes granules; Mi, mitochondria; Mpt, middle piece of the tail; N, nucleus; n, sperm nuclei; nu, nucleolus; S, spermatozoa; Sc, secretory cell; Sm, secretory material; Sv, secretory vacuoles; Tj, tight junction; Sg, shell glands

Fox (1956) observed the presence of ciliated ducts that connect the infundibular lumen to compound alveolar glands or sperm receptacles in *Thamnophis sirtalis* (Natricidae) and *T. elegans terrestris*. The tubular ciliated glands of *E. miliaris* are similar to the ducts that assist the transport of spermatozoa to the receptacles in *Thamnophis.* The receptacles described herein for *E. miliaris* are similar to observed in *T. sirtalis* and *T. elegans terrestris*. Both SSr characterise by the presence of only secretory cells and absence of ciliated cells. Because females of *E. miliaris* may store sperm throughout the year, including during the pregnancy period, they may be able to produce multiple clutches at different seasons of the year regardless of mating during autumn. Multiple clutches in *E. miliaris* (Di-Bernardo, 1998) and *Erythrolamprus semiaureus* (Dipsadidae) (Bonfiglio, 2007) from the southern region of Brazil have already been described previously. Sperm remains stored in infundibular receptacles in post-partum females of *P. patagoniensis,* and it can explain their ability to produce multiple clutches in different seasons of the year (Rojas et al., 2015). The parallel alignment of spermatozoa inside the receptacles of *E. miliaris* is a common pattern for snakes (except for *S. pygaea*) and may be related to reduction in energy expenditure (Aldridge, 1992; Fox, 1956; Rojas et al., 2015; Siegel & Sever, 2008*a*). However, the relation between the epithelial cells of sperm receptacles and the stored spermatozoa could not be observed by TEM, maybe due to some problem with sampling.

The glandular uterus of *E. miliaris* showed an increase in the secretory activity of the epithelium and shell glands in vitellogenic females. The production of electron lucent secretory vacuoles by the uterine epithelial cells associated to the strong positivity to PAS in *E. miliaris* suggests the secretion of neutral carbohydrates (Siegel et al., 2011). An increase in the secretory activity usually occurs under the influence of oestrogen hormones as described for *Crotalus durissus terrificus* (Viperidae) (Almeida-Santos et al., 2004). This secretion may possibly have a role in the formation of the shell membrane as in *T. sirtalis* and *P. patagoniensis* (Hoffman, 1970; Rojas et al., 2015). Histochemical analyses (PAS and CB) in *E. miliaris* confirm the production of glycoproteins by shell glands in vitellogenic females. The cells of the shell glands of vitellogenic females show an increase in the production of slightly electron dense vacuoles and the occurrence of rough endoplasmic reticulum, which

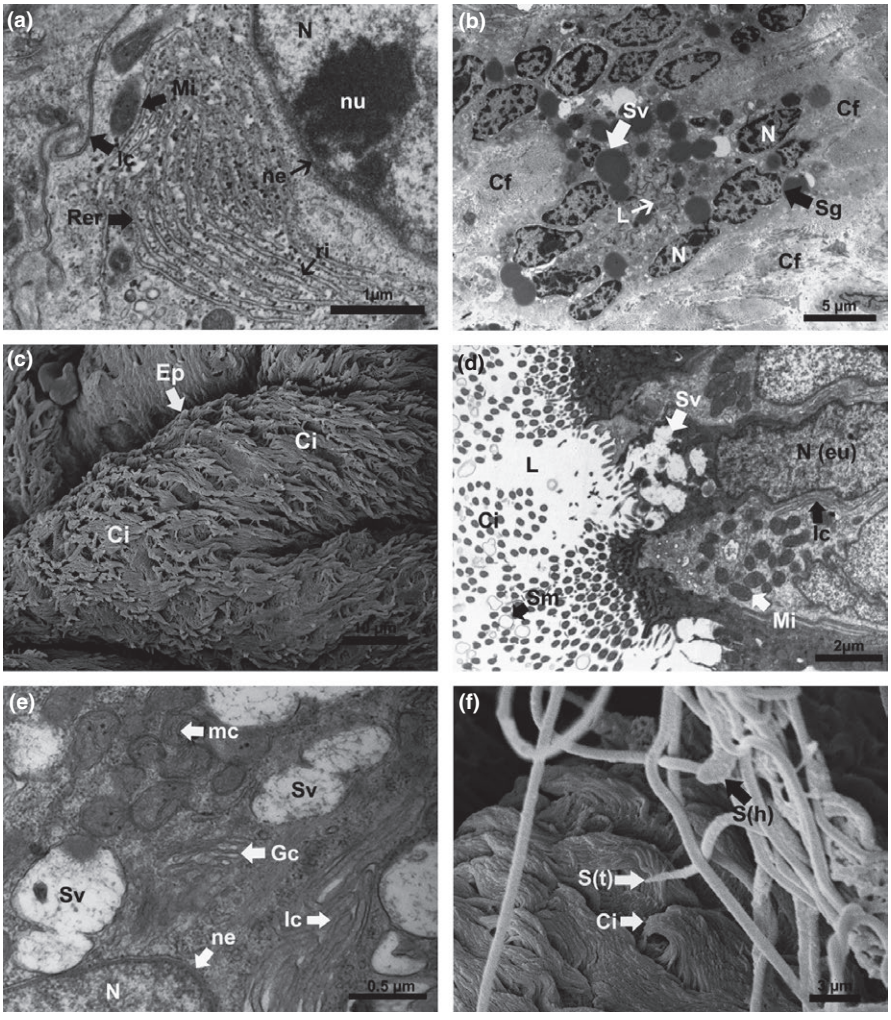

FIGURE 5 Ultrastructural characteristics of the glandular uterus and non-glandular uterus in female of *Erythrolamprus miliaris*. (a) Higher magnification of the glandular uterine cell during spring. (b) Atrophy of the shell glands in pregnant female. (c) Scanning electron microscopic of the non-glandular uterus epithelium. (d) Transmission electron micrograph of the non-glandular uterus with increased secretory activity in autumn and spring. (e) Higher magnification in the cytoplasm of the secretory cells of the non-glandular uterus. (f) Scanning electron microscopic of the non-glandular uterus with spermatozoa in the lumen during autumn. Cf, collagen fibre; Ci, cilia; Ep, epithelium; Gc, Golgi complex; Ic, intercellular canaliculi; L, lumen; mc, mitochondrial cristae; Mi, mitochondria; N, nucleus; N (eu), euchromatic nucleus; ne, nuclear envelope; nu, nucleolus; Rer, rough endoplasmic reticulum; ri, ribosomes; S (h), spermatozoa head; S (t), spermatozoa tail; Sg, shell glands; Sm, secretory material; Sv, secretory vacuoles

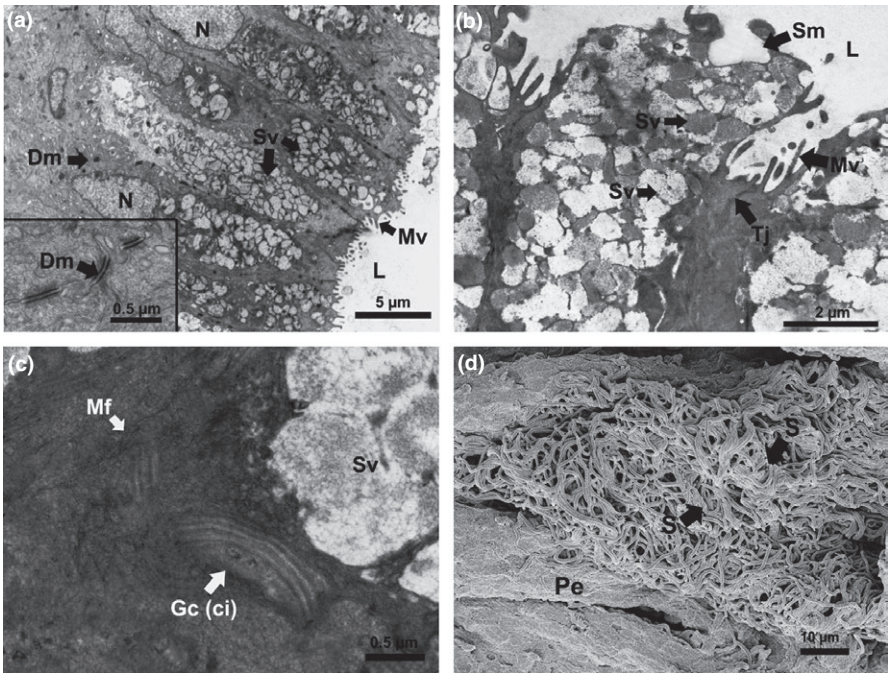

FIGURE 6 General ultrastructure of the pouch in female of *Erythrolamprus miliaris*. (a) Pouch epithelium during reproductive cycle. Inset: desmosome. (b) Apocrine secretion by the pouch epithelium. (c) Higher magnification of the pouch secretory cells. (d) Spermatozoa in the pouch lumen. Dm, desmosome; Gc (ci), Golgi cisterns; L, lumen; Mf, microfilaments; Mv, microvilli; N, nucleus; S, spermatozoa; Sm, secretory material; Sv, secretory vacuoles; Tj, tight junction; Pe, pouch epithelium

is an indicative of the production of protein used in the formation of the shell membrane. This model has already been described for *Diadophis punctactus* (Colubridae) (Perkins & Palmer, 1996), *Lacerta vivípara* (Lacertidae) (Heulin et al., 2005) and *P. patagoniensis* (Rojas et al., 2015). According to Hoffman (1970), the uterine glands of *T. sirtalis* are responsible for the production of pseudokeratin, the main component of the membrane shell in this species. The atrophy of uterine glands in pregnant females of *E. miliaris* suggests that the material secreted by glandular cells is used for the production of the membrane shell. Most oviparous snakes have a glandular uterus with a higher concentration of shell glands than viviparous species, which influences the thickness of the shell membrane (Blackburn, 1998; Heulin et al., 2005).

The presence of long folds and deep furrows is characteristic of the non-glandular uterus region in every species studied to date. These furrows may be sperm storage sites for some species (Almeida-Santos & Salomão, 1997; Barros et al., 2014b; Rojas et al., 2015; Siegel et al., 2011). The existence of sperm in the non-glandular uterus only in April for *E. miliaris* indicates a temporary passage of spermatozoa through this oviductal portion, which does not seem to be a sperm storage site for this species. On the other hand, the non-glandular uterus is a long-term sperm storage site for other snake species such as *C. durissus* (Almeida-Santos & Salomão, 1997; Barros et al., 2012), *Bothrops sp.* (Viperidae) (Almeida-Santos & Salomão, 2002; Barros et al., 2014a,b) and *P. patagoniensis* (Loebens et al., 2017; Rojas et al., 2015). Different types of epithelium may characterise this oviductal region. For most snakes, the epithelium of the non-glandular uterus is simple columnar, composed mainly of ciliated cells interspersed with secretory cells (Almeida-Santos & Salomão, 1997; Fox, 1956; Sever et al., 2000; Siegel et al., 2011). However, some snake species like *Tantilla coronata* (Colubridae), *Bothrops erythromelas* and *P. patagoniensis* exhibit pseudostratified epithelium in the non-glandular uterus, similar to that described to *E. miliaris* (Aldridge, 1992; Barros et al., 2014b; Rojas et al., 2015). Positive reactions for neutral carbohydrates have already been described for *T. sirtalis*, *D. punctactus* and *P. patagoniensis* (Fox, 1956; Perkins & Palmer, 1996; Rojas et al., 2015). The increase in this secretion during vitellogenesis and mating season indicates a hormonal influence during the reproductive cycle. Females of *C. d. terrificus* show high levels of estradiol during vitellogenesis (Almeida-Santos et al., 2004). Ultrastructural analyses of the non-glandular uterus epithelium have already been conducted for *S. pygaea* (Sever et al., 2000), *C. durissus*, *Bothrops jararaca* (Almeida-Santos, 2005) and *A. piscivorus* (Siegel & Sever, 2008b). The morphology of ciliated cells characterised by many mitochondria associated to basal body where cilia are anchored is similar to the morphology observed in the non-glandular uterus for other snake species (Almeida-Santos, 2005; Sever et al., 2000; Siegel & Sever, 2008b). The synthesis and merocrine secretion of flocculent material by secretory cells are similar to *S. pygaea* (Sever & Ryan, 1999).

The microscopical characteristics of the pouch of *E. miliaris* (uniformly secretory epithelium) are similar to most snake species studied to date (Rojas et al., 2015; Sanchez-Martínez, Ramírez-Pinilla, & Miranda-Esquivel, 2007; Siegel et al., 2011; Uribe, González-Porter, Palmer, & Guillette, 1998). The pseudostratified epithelium in the pouch of *E. miliaris* was described previously for other species as *Macroprotodon cucullatus* (Colubridae) and *P. patagoniensis* (Rojas et al., 2015; Siegel et al., 2011). A unique characteristic of the pouch epithelium of *E. miliaris* is the high number of cell junctions or desmosomes. The function of these structures is to maintain the cell–cell adhesion, avoiding tissue disarrangement caused by mechanical stress or stretch during oviposture. The most common organelle in the secretory cells of the pouch epithelium of *E. miliaris* is the Golgi complex, which suggests a continuous production of neutral carbohydrates (mucus) for lubrication and maintenance of mucosal moisture. Various authors (Gabe & Saint-Girons, 1965; Rojas et al., 2015; Uribe et al., 1998) have already described constant positive reactions for PAS and AB in the pouch epithelium. The release of secretory material through a merocrine process is similar to that observed for *A. piscivorus* (Siegel & Sever, 2008b). The presence of spermatozoa in the pouch lumen of *E. miliaris* only during April confirms the existence of a single mating season for this species. Thus, the production of multiples clutches throughout the year in *E. miliaris* proves that sperm storage is an obligatory reproductive tactic.

In summary, the morphofunctional alterations in the oviduct of *E. miliaris* during the reproductive cycle are directly influenced by mating season, vitellogenesis and sperm storage. This is the first description of sperm storage receptacles in females of *E. miliaris*, and it is the second report for a dipsadid snake from the Neotropical region (see Loebens et al., 2017; Rojas et al., 2015). The characterisation of the morphofunctional bases of the reproductive tract in different species of snakes from the Neotropical region may help to elucidate anatomical and reproductive patterns shared by specific lineages.

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